

A method for immobilization of lipoxygenase¹

Dennisse Parra-Diaz, Dorothy P. Brower, Marjorie B. Medina
and George J. Piazza²

Eastern Regional Research Center, ARS, USDA, 600 E. Mermaid Lane, Philadelphia,
PA 19118, U.S.A.

A commercial preparation of soybean [*Glycine max* (L.) Merr.] lipoxygenase (EC 1.13.11.12), an enzyme that catalyses the formation of fatty acid hydroperoxides, was covalently immobilized on a commercially available carbonyldi-imidazole activated support. The degree of protein loading on to the support and the subsequent activity of immobilized lipoxygenase were found to be independent of the pH at which coupling was performed. Dialysis of lipoxygenase prior to immobilization did not enhance the coupling yield. As protein bound to the support increased, the specific activity of lipoxygenase decreased. The reusability of immobilized lipoxygenase was tested in an aqueous buffer and in an octane/aqueous buffer medium using linoleic acid as the substrate. In aqueous buffer the immobilized preparation retained its activity even after seven cycles, whereas in the octane/buffer medium the activity of the immobilized preparation decreased to 60% of its original activity after seven cycles. The optimal temperature for hydroperoxide formation was 15°C, with hydroperoxide yields decreasing at higher temperatures. Storage of immobilized lipoxygenase at 5°C resulted in a loss from the support of approx. 5% of the protein in 25 days, but none thereafter. Lipoxygenase activity was stable at 5°C, decreasing by only 5% in 6 months. The stability of immobilized lipoxygenase at 15°C in aqueous buffer was approx. 10-fold greater than that of unbound lipoxygenase. The results show that immobilized lipoxygenase can be used in aqueous media as well as those containing organic solvent.

Fatty acid hydroperoxides are of continuing interest to industry and the medical-research community because reduced derivatives of fatty acid hydroperoxides could be used in a variety of industrial products [1,2], and fatty acid hydroperoxides and their derivatives are important mediators of a number of biological processes [3,4]. Hence an interest is emerging in the regio- and enantio-selective synthesis of fatty acid hydroperoxides and their derivatives. One route to this class of compounds is to use the enzyme lipoxygenase (LOX³, EC 1.13.11.12) to introduce the hydroperoxide functionality into a polyunsaturated fatty acid. It is known that LOX obtained from a variety of natural sources shows nearly strict positional specificity for the carbon atom that becomes

¹ Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

² To whom correspondence should be addressed.

³ Abbreviations used: HPOD, hydroperoxyoctadecadienoic acid; IMM-LOX, immobilized lipoxygenase; LA, linoleic acid; LOX, lipoxygenase.

oxidized, and that the hydroperoxide functionality is introduced in a stereospecific manner [5]. Any industrial process in which LOX is envisioned as a catalyst for synthesis requires that LOX be immobilized so that it can be recycled in bioreactors. Also, as a result of immobilization, there is often increased enzyme stability, enhancement of process control and elimination of the contamination of products with spent protein. Besides obtaining an immobilized preparation *per se*, it was of interest to obtain one in which the support was stable in organic solvents, because ways of using LOX to functionalize hydrophobic substrates are a long-range goal of this laboratory.

In contrast with the lipase literature [6], the literature on LOX immobilization procedures is very limited [7–11]. Soybean LOX-1 has been covalently immobilized on oxirane acrylic beads, but the coupling reaction time was 60 h [7], and the immobilized preparation lost activity after only one cycle. Another matrix on which soybean LOX-1 was immobilized was a CNBr-activated Sepharose, but this approach required a purification step for LOX prior to its immobilization [8]. A functional bioreactor was prepared using this immobilized LOX preparation, but such a reactor would have a limited lifetime, owing to the instability of the isourea linkage between LOX and Sepharose [9]. LOX was immobilized on CNBr-activated agarose, but this preparation's stability for recycling was not investigated [10]. Continuous experiments using LOX adsorbed through hydrophobic interactions, covalently bound by CNBr and physically adsorbed, then cross-linked by glutaraldehyde, revealed that LOX was still labile with a half-life of several hours [11].

In our initial approaches we tried non-covalent immobilization of LOX on to cation-exchange and anion-exchange resins, a hydrophobic inclusion support, a hydrophobic surface support and an absorption support. In each case subsequent activity assays showed that fatty acid oxidation was low or undetectable when oxygenations were conducted in organic solvents. Since it was not possible to determine whether the low activity was due to the presence of organic solvent or to the nature of the support, it was decided that a covalent immobilization procedure should be sought, since then a meaningful comparison of enzymic activity in both aqueous and organic media could be made.

In this study an optimized procedure for the immobilization of LOX on to a commercially available carbonyldi-imidazole activated matrix was developed. This matrix is compatible with organic and aqueous media. The urethane linkage formed by this procedure is about 20-fold more stable than the N-substituted isourea linkage formed during protein immobilization on to CNBr-activated matrices [9].

Materials and methods

Soybean LOX (Lipoxidase, type 1-B), linoleic acid (LA), protein standard and cumene hydroperoxide were purchased from Sigma (St. Louis, MO,

U.S.A.). Reacti-Gel (6X) was purchased from Pierce (Rockford, IL, U.S.A.). T.l.c. plates were purchased from Analtech (Newark, DE, U.S.A.). The sodium salt of Xylenol Orange was purchased from Aldrich (Milwaukee, WI, U.S.A.). Protein Reagent was purchased from Bio-Rad (Richmond, CA, U.S.A.). Water was purified to a resistance of 18 mΩ·cm using a Barnstead (Dubuque, IA, U.S.A.) NANOpure system. All other reagents were of the highest purity available.

LOX immobilization

A 1,1'-carbonyldi-imidazole-activated 6% cross-linked beaded agarose with an activation level greater than 50 μmol/ml of gel, termed Reacti-Gel, was used for immobilizing LOX. The optimized procedure was as follows. After the Reacti-Gel (1 ml) reached room temperature, it was placed in a 10.0 ml disposable polypropylene column fitted with a porous polyethylene disk (Pierce, Rockford, IL, U.S.A.). The acetone in which the Reacti-Gel was suspended was removed from the gel without allowing the gel to dry completely. The gel was washed three times with 5.0 ml of water and was mixed with 3 ml of a 2.0 mg/ml solution of LOX in 0.2 M borate buffer, pH 9.0, and the mixture was incubated with gentle agitation at 4°C. During coupling, the pH of the gel/enzyme mixture was adjusted three times to the original pH. After 42 h the gel and buffer were separated by filtration, and the gel cake was then washed with 5 ml of 0.2 M borate buffer, pH 9.0. The remaining active sites on the gel were blocked by incubating the gel cake with 3.5 ml of 2 M Tris buffer, pH 8.0, for 30 min at room temperature. Finally, the gel cake was washed three times with 10 ml of cold water and stored at 4°C in 0.1 M phosphate buffer, pH 7.0, containing 0.9% NaCl, 0.05% BSA and 0.02% NaN₃. This buffer will be referred to as 'storage buffer'. In those experiments in which the pH of the coupling buffer was varied, a mixture of 0.2 M borate, EDTA and Tricine was used as the buffer.

Percentage protein load on Reacti-Gel

Prior to incubation with Reacti-Gel, the amount of protein in the LOX/borate buffer mixture was estimated using Bio-Rad Protein Reagent. Sigma albumin and globulin protein standards were used for calibration. After coupling was complete, the gel was separated from the buffer mixture and was washed as described in the immobilization procedure. The volume of the combined buffer and wash was measured, and the amount of recovered protein determined. The amount of protein bound to the gel was estimated from the difference between the amount of added and recovered protein. The percentage protein load is this value divided by the amount of protein initially added multiplied by 100.

Hydroperoxyoctadecadienoic acid (HPOD) formation in aqueous and organic media

For assays performed in aqueous media, the following is a typical procedure. Storage buffer was removed from the immobilized LOX (IMM-LOX)

by filtration. The IMM-LOX was washed with two 3.0 ml aliquots of 0.2 M borate buffer, pH 9.0. IMM-LOX (0.59 g containing 3 mg of protein) was added to 40 mg of LA that had been previously suspended by sonication for 30 min in 20 ml of 0.2 M borate buffer, pH 9.0. The reaction was allowed to proceed at 15 °C, while the reaction medium was agitated at 250 rev./min. After a specified reaction time, IMM-LOX was filtered from the reaction mixture, washed with two 10 ml aliquots of borate buffer and placed in storage buffer at 5 °C or returned to a fresh suspension of LA. An aliquot from the reaction solution was assayed for hydroperoxide content.

For assays in organic solvent, 40 mg of LA was dissolved in 15.0 ml of water-saturated octane in a 125 ml glass-stoppered Erlenmeyer flask. After washing IMM-LOX with 0.2 M borate buffer, pH 9.0, 0.3 g IMM-LOX containing 1.5 mg of protein and 187 μ l of 0.2 M borate buffer, pH 9.0, were added to the LA solution. The reaction was allowed to proceed at 15 °C, while the reaction medium was agitated at 250 rev./min. After a specified reaction time the gel was filtered from the reaction mixture, and the IMM-LOX was washed with two 10 ml aliquots of borate buffer which were similarly filtered. The pH of the water layer was lowered to 3.0, and the aqueous and octane layers were separated. The aqueous layer was extracted three times with 5 ml of diethyl ether. The ether and the octane fractions were combined, and an aliquot was taken for hydroperoxide assay.

HPOD assay

HPOD was measured spectrophotometrically using the Xylenol Orange method [12]. The Xylenol Orange reagent was composed of 100 μ M Xylenol Orange, 250 μ M ammonium ferrous sulphate, 25 mM H₂SO₄ and 4 mM 2,6-di-*t*-butyl-4-methylphenol in methanol/water (9:1, v/v). The reagent (2.0 ml) was added to the sample (10–50 μ l), and the volume was raised to 2.1 ml with ethanol. The assays were performed at room temperature for 45 min, and the absorbance at 560 nm was measured versus a blank that was a mixture of 2.0 ml of the Xylenol Orange reagent and 100 μ l of ethanol. Freshly diluted commercial cumene hydroperoxide was used for preparing a calibration curve of the dye reagent each day.

T.l.c. analysis was performed on each reaction mixture as a check on the hydroperoxide levels given by the Xylenol Orange method and also to determine if anaerobic-by-product formation and/or decomposition of HPOD was occurring [13]. Silica-gel-HL t.l.c. plates (10 cm \times 10 cm) were dipped in 5% boric acid in methanol and allowed to air-dry prior to spotting. The t.l.c. plates were developed sequentially in the following solvent systems: diethyl ether/benzene/ethanol/acetic acid (200:250:10:1, by vol.); air-drying; iso-octane/diethyl ether/acetic acid (25:25:1, by vol.). Hydroperoxides were revealed by charring after spraying the t.l.c. plates with 60% H₂SO₄. The R_F values of LA, HPOD and hydroxyoctadecenoic (ricinoleic) acid were 0.77, 0.62 and 0.53 respectively. Since the t.l.c.

system cannot differentiate between fatty acids having differing degrees of unsaturation, the R_F value of hydroxyoctadecadienoic acid, the reduced product of HPOD, is also 0.53.

Results and discussion

The immobilization of LOX was investigated over the pH range 7.0–10.5 at pH-unit intervals to determine whether the loading of LOX would change. It was found that the amount of protein bound to the gel was identical within experimental error at all pH values studied, despite the manufacturer's recommendation that pH values greater than 8.5 be used for coupling. When the various IMM-LOX preparations were assayed at pH 9.0, no difference in their ability to catalyse the formation of HPOD was noted.

Since the LOX used in this study was a commercial preparation with added stabilizer, the preparation was dialysed prior to immobilization to determine whether the stabilizer interfered with immobilization. The percentage protein load on the gel from samples that were dialysed was $78 \pm 5\%$ (S.E.M., $n=8$), whereas that from samples that were not dialysed was $76 \pm 2\%$ (S.E.M., $n=16$). Thus it was concluded that the stabilizer did not interfere with LOX immobilization. In addition, t.l.c. analysis of the reaction product obtained from the oxidation of LA using IMM-LOX showed that dialysis did not affect HPOD formation.

The influence of the concentration of protein used in the coupling procedure upon the percentage protein load and the yield of HPOD was investigated (Table 1). The percentage protein load on the gel was approx. 70%, and very little change in the percentage of protein bound was noted when the amount of added protein was varied from 5.1 to 34.1 mg of protein/g of gel. The best yields of HPOD were obtained with IMM-LOX preparations derived from coupling media that contained the lowest amount of protein. Because protein levels were kept constant, these assays also contained the highest amount of gel. That the gel itself did not promote HPOD formation was shown by the following experiments. De-activated gel (gel that was treated with Tris buffer) containing no LOX did not promote non-enzymic oxidation of LA. Moreover, when de-activated gel that contained no protein was added to IMM-LOX, HPOD formation was not enhanced.

Unbound LOX was assayed using the same reaction conditions as those given in footnote^a of Table 1, and it was found that 22.8 ± 0.5 and $27.1 \pm 0.7 \mu\text{mol}$ (S.E.M., $n=3$) of HPOD formed when 1.5 and 3.0 mg of protein respectively were added to the assays. Thus it is concluded from comparison of these values with those listed in Table 1 that the immobilization of LOX results in an apparent stimulation of its activity.

The degree of protein loss with time from IMM-LOX was determined. After removal of IMM-LOX from the storage buffer, IMM-LOX was rinsed with water, placed into water, and stored at 5 °C. At a specified

Table 1 Effect of the amount of protein added during coupling on the amount of HPOD formed

[Protein] during coupling (mg of protein/g of gel)	IMM-LOX in assay		HPOD formed in assay ^a (μ mol)
	Protein (mg)	Gel (g)	
5.1	1.5	1.47	49.2 \pm 4.6 ^b
10.2	1.5	0.73	40.7 \pm 5.4
22.7	1.5	0.33	36.1 \pm 2.4
34.1	1.5	0.22	30.3 \pm 2.4
5.1	2.3	2.25	60.8 \pm 1.8
10.2	2.3	1.12	50.3 \pm 4.1
22.7	2.3	0.51	34.7 \pm 3.0
34.1	2.3	0.34	36.6 \pm 2.2

^aIn addition to IMM-LOX, assay contained 40 mg of LA, 15 ml of water-saturated octane and 0.187 ml of 0.2 M borate buffer, pH 9.0. The reaction time was 3 h, and the reaction temperature was 15 °C.

^bData are the mean \pm S.E.M. of four repetitions.

Table 2 Typical results of sequential washings of IMM-LOX to determine protein leakage over time

Time after coupling (days) ^a	Protein in water rinses	
	(μ g)	(%) ^b
13	207 ^c	2.2
13	72	0.8
13	117	1.3
25	59	0.6
25	56	0.6
40	nd ^d	
40	nd	
40	nd	

^aReacti-Gel (1 g) contained 9.29 mg of protein.

^bBased upon total protein in IMM-LOX.

^cProtein in water rinses of IMM-LOX; the mean volume of each rinse was 5.6 ml.

^dnd, not detected.

time IMM-LOX was removed from the water and washed once or twice more with water by shaking it for 30 s. The protein levels in the storage and wash water were then determined (Table 2). Over 25 days storage the total protein that leaked from the gel was 5.5%, and beyond 25 days no further protein could be detected in the storage water or water washes.

Table 3 shows that IMM-LOX can be re-used in aqueous buffer media and in media containing organic solvent. In aqueous buffer, the activity of IMM-LOX increased slightly in the first few cycles, reaching maximum activity during the third cycle. Thereafter its activity slowly declined, so that, at the seventh cycle, its activity was approximately equal to that of the initial preparation. In octane/aqueous buffer mixtures, however, the activity of IMM-LOX showed a gradual decrease with each re-use; at the

Table 3 Activity of IMM-LOX with repeated use

Cycle number	Relative activity (%)	
	Recycled in aqueous buffer ^a	Recycled in octane/ aqueous buffer ^b
1 ^c	100 ± 2	100 ± 7
2	116 ± 3	102 ± 5
3	123 ± 3	86 ± 3
4	121 ± 5	79 ± 3
5	114 ± 4	72 ± 4
6	108 ± 3	59 ± 3
7	104 ± 4	60 ± 3

^aAssays were performed for 1 h at 15 °C in 20 ml 200 mM borate buffer, pH 9.0, containing 40 mg of LA and 0.59 g of IMM-LOX having 3 mg of bound protein; at 100% activity, the rate of conversion of LA into HPOD was 0.63 μ mol/min per mg of protein; results are the means \pm S.E.M. for four repetitions.

^bAssays were performed for 1 h at 15 °C in 15 ml of water-saturated octane containing 6 ml of 200 mM borate buffer, pH 9.0, 40 mg of LA and 0.59 g IMM-LOX having 3 mg of bound protein; at 100% activity, the rate of conversion of LA into HPOD was 0.47 μ mol/min per mg of protein; results are means \pm S.E.M. for four repetitions.

^cThe storage buffer was removed from IMM-LOX by filtration, and the IMM-LOX was washed with two aliquots of 3 ml of 0.2 M borate buffer, pH 9.0, before its addition to the assay.

seventh cycle, 60% of the initial activity was retained. Thus the results show that IMM-LOX could be used in an organic-buffer medium or in a purely aqueous medium.

The amount of HPOD formed from LA was monitored at different temperatures using IMM-LOX as catalyst. Representative data from 3 h reactions in media containing octane are shown in Figure 1. The best temperature was 15 °C. The rate of HPOD formation decreased at 7 °C, as expected from the normal effect of lower temperature upon an enzymically catalysed reaction. However, contrary to expectations, HPOD formation decreased at temperatures higher than 15 °C. That this decrease was due mostly to the decomposition of HPOD and/or anaerobic byproduct formation due to decreased oxygen solubility at higher temperature [14], rather than heat-instability of IMM-LOX, was indicated by the following. T.l.c. analysis of the products generated at higher temperatures showed extensive decomposition, as evinced by the presence of materials that had lower R_F values than HPOD. At 23 °C and above, the yield of HPOD plateaued at approx. 40% of theoretical and then gradually decreased with time. When IMM-LOX was recovered from the higher-temperature reactions and used again at 15 °C, the amount of HPOD generated was the same as that formed by a preparation of IMM-LOX that had never been exposed to high temperature.

The stability of IMM-LOX was determined in two different ways. The activity of IMM-LOX in storage buffer at 5 °C was periodically measured. Over a 6 month period, approximately 95% of the IMM-LOX activity was retained. That this stability is due to the immobilization procedure *per se* was shown by comparing the enzymic stability of unbound LOX with that of IMM-LOX at 15 °C in 100 mM Tricine, pH 9.0. As shown in Figure 2(a),

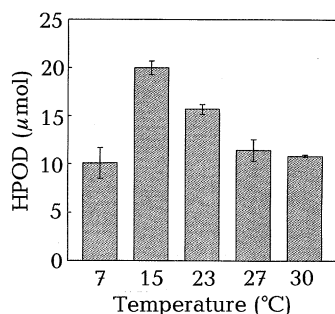


Figure 1

The influence of temperature on HPOD formation catalysed by IMM-LOX. Assays were performed for 3 h in 15 ml of water-saturated octane containing 0.187 ml of 200 mM borate buffer, pH 9.0, 40 mg of LA and 0.54 g of IMM-LOX having 2.4 mg of bound protein.

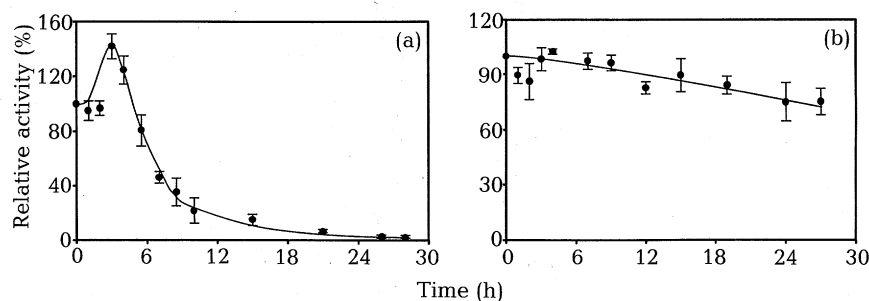


Figure 2

Stability of unbound LOX (a) and IMM-LOX (b) at 15°C. LOX (2 mg of powder containing 1 mg of protein) or IMM-LOX (0.194 g of gel containing 1 mg of protein) was dispersed in 14.5 ml of Tricine buffer, pH 9.0. At the indicated time, 40 mg of LA in 100 μ l of ethanol was added. The reaction was allowed to proceed for 15 min.

unbound LOX showed maximal activity after 3 h, and thereafter its activity rapidly decayed with a half-life of approx. 7 h. In contrast, as shown in Figure 2(b), IMM-LOX retained about 75% of its activity at 28 h. The half-life of IMM-LOX was estimated to be 75 h by replotting the activity data on a logarithmic scale and using a linear least-squares regression fit [15]. Thus IMM-LOX is approx. 10-fold more stable than unbound LOX.

Although the studies presented here were conducted with readily available soybean LOX and LA, the optimized immobilization procedure should be useful for stabilizing LOX from more exotic and/or expensive sources. Here retention of activity is crucial for the production of hydroperoxides, particularly those of arachidonate, which are currently of immense scientific interest.

References

- 1 Emken, E. M. and Dutton, H. J. [1971] *J. Am. Oil Chem. Soc.* **48**, 324-329
- 2 Cardillo, R., Fronza, G., Fuganti, C., Grasselli, P., Mele, A., Pizzi, D., Allegrone, G., Barbeni, M. and Pisciotta, A. [1991] *J. Org. Chem.* **56**, 5237-5239
- 3 Yamamoto, S. [1992] *Biochim. Biophys. Acta* **1128**, 117-131
- 4 Nicolaou, K. C., Ramphal, J. Y., Petasis, N. A. and Serhan, C. N. [1991] *Angew. Chem. Int. Ed. Engl.* **30**, 1110-1116
- 5 Kühn, H., Schewe, T. and Rapoport, S. M. [1986] *Adv. Enzymol. Relat. Areas Mol. Biol.* **58**, 273-311
- 6 Malcota, F. X., Reyes, H. R., Garcia, H. S., Hill, C. G., Jr. and Amudson, C. H. [1990] *J. Am. Oil Chem. Soc.* **67**, 890-910
- 7 Maguire, N. M., Mahon, M. F., Molloy, K. C., Read, G., Roberts, S. M. and Sik, V. [1991] *J. Chem. Soc. Perkins Trans. 1* 2054-2056
- 8 Laakso, S. [1982] *Lipids* **17**, 667-671
- 9 Hearn, M. T. W., Harris, E. L., Bethell, G. S., Hancock, W. S. and Ayers, J. A. [1981] *J. Chromatogr.* **218**, 509-518
- 10 Grossman, S., Trop, M., Budowski, P., Perl, M. and Pinsky, A. [1972] *Biochem. J.* **127**, 909-910
- 11 Yamane, T. [1982] in *Enzyme Engineering* (Chibata, I., Fukui, S. and Wingard, L. B. J., eds.), vol. 6, pp. 141-142, Plenum, New York
- 12 Jiang, Z.-Y., Woollard, A. C. S. and Wolff, S. P. [1991] *Lipids* **26**, 853-856
- 13 Graveland, A. [1973] *Lipids* **8**, 606-611
- 14 Gardner, H. W. [1991] *Biochim. Biophys. Acta* **1084**, 221-239
- 15 Pitcher, W. H., Jr. [1975] in *Immobilized Enzymes for Industrial Reactors* (Messing, R. A., ed.), pp. 151-199, Academic Press, New York